

Assessment of Human Lung Macrophages After Exposure to Multi-Walled Carbon Nanotubes Part I. Cytotoxicity

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Due to the widespread production and use of carbon nanotubes in almost every area of science (i.e., drug delivery, biosensors, fuel cells and thermal management systems), they are receiving considerable attention for their novel mechanical, electrical and chemical properties. At this time of high exposure potential, it is critical to ascertain the biological impact of these materials on likely target organs, tissues and cells, such as those of the lung. The aim of this study was to evaluate the degree of cytotoxicity to human lung macrophage (U937) cells after exposure to unpurified or acid-purified multi-walled carbon nanotubes. Cells were incubated with multi-walled carbon nanotubes and assessed for cytotoxicity, generation of reactive oxygen species, morphological changes and uptake. The results demonstrate that multi-walled carbon nanotubes can accumulate in human lung macrophage cells to different degrees based on their surface chemistry. MWNT-COOH reduces cell viability in a dose-dependent manner under these experimental conditions (5–50 $\mu\text{g/ml}$, 2–24 h). However, images of individual cells demonstrate morphological changes at low concentrations. Therefore, before nanomaterials are fully accepted and integrated into biological systems, they will continue to undergo further scrutiny at various stages of their processing (i.e., before and after purification) and with models ranging from simple to complex (i.e., cells vs. whole animals) to gain a better understanding between their physicochemical properties and bio-effects.

Keywords: U937 Cell, MWNTs, MWNT-COOH, ROS.

1. INTRODUCTION

Carbon nanotubes consist of carbon hexagons arranged in a concentric manner with ends capped by fullerene-like structures containing pentagons.³¹ They can be further sub-classified based on the number of walls and are termed single-walled (SWNTs), double-walled (DWNTs), multi-walled (MWNTs), etc. although many other forms and structures such as carbon nanohorns, nanodiamonds, etc. exist. Due to the atomic arrangement of CNTs, they are characterized by high aspect ratios, high strengths, high thermal and electrical conductivities, and the ability to be chemically functionalized or filled. It has been determined that the properties of CNTs are strongly dependent upon their inherent semi-conductive or conductive structure; the presence of impurities such as residual catalysts or other

forms of carbon; and defects from chemical or thermal processing for purification and functionalization.

Phenomenon such as enhanced chemical reactivity, permeability, and the profound effect of surface chemistry are all expected to influence the cellular dynamics of nanomaterials.¹ In the case of carbon nanotubes (CNTs), most studies have focused on organs such as the lungs.^{2–7} Other studies have utilized macrophages in cell culture, which are responsible for removing foreign debris and inflammatory responses.^{8–11} Previous studies in our laboratory¹² and others¹⁰ have shown that nanoparticles can alter the phagocytic response of macrophages, which may have implications in disease conditions. Therefore, further investigation into the factors that influence nanomaterial cytotoxicity, such as purification procedures and resultant surface chemistry continue to be elucidated.^{13–15} Alveolar macrophages are found in the alveolar sacs deep within the lungs. These cells are the first line of

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immunological defense against inhaled particles and serve as a good model to investigate how inhaled particles can adversely affect cell function and lead to degradation of health.³⁰ In this study, we chose the human lung macrophage cell line (U937) as a model to investigate the potential cytotoxicity of unpurified multi-walled carbon nanotubes (MWNTs) and acid purified multi-walled carbon nanotubes (MWNT-COOH) by detecting the generation of reactive oxygen species (ROS), morphological changes and cell uptake.

2. RESULTS AND DISCUSSION

The unpurified MWNTs and acid-purified MWNT-COOH were characterized with transmission electron microscopy (TEM), air thermogravimetric analysis (TGA), Raman spectroscopy and X-ray photoelectron spectroscopy (XPS). There was visual evidence for a reduction in nanotube length after the acid purification and sonication treatment compared to the original unpurified sample (data not shown). However, once the MWNTs were introduced into aqueous solutions, the nanotubes behaved in an agglomerated fashion. We previously demonstrated that large MWNT agglomerates (microns in size) were present in 1 mg/ml stock solutions, which were only briefly sonicated for 10 seconds.¹⁶ In this study, immediately after dosing cells in culture with a low 5 $\mu\text{g/ml}$ concentration of MWNTs or MWNT-COOH, there was visual evidence for large agglomerated bundles of MWNTs with sizes >50 microns (data not shown) in comparison to better dispersion of MWNT-COOH. However, differences in MWNT length (3.5 microns vs. 12 microns measured with TEM) due to short or extended sonication times have not been shown to influence subsequent cytotoxicity.⁷ Therefore, in addition to visualization of primary MWNT structure and overall dispersion, metal catalyst impurity content and surface chemistry were considered primary characteristics for each MWNT sample in this study.

The TGA data for the unpurified MWNTs (Fig. 1) revealed ~11 wt% orange-colored residue after being heated above 730 $^{\circ}\text{C}$, most likely due to the presence of Fe catalyst residues that became converted to Fe_2O_3 upon thermal oxidation. The MWNT-COOH sample showed a reduced Fe catalyst residue content of ~1.9 wt% above 730 $^{\circ}\text{C}$, which indicates that the acid treatment removed most of the Fe catalyst residue. These results for impurity content were similar to our previous studies using the same MWNTs, which displayed 8 wt% Fe for unpurified MWNTs compared to 0.49 wt% for acid-purified MWNTs¹⁶ when analyzed with inductively-coupled plasma-optical emission spectroscopy (ICP-OES).

The Raman spectra of the MWNT sample before and after acid purification showed an increase in the disorder (D) band (1350 cm^{-1}) for the MWNT-COOH sample suggesting that some surface damage due to oxidation

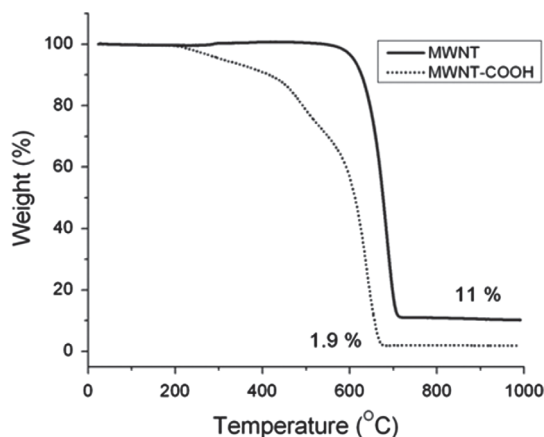


Fig. 1. Characterization of MWNTs and MWNT-COOH with TGA showing removal of Fe catalyst residues after acid purification.

occurred (data not shown). X-ray photoelectron spectroscopic (XPS) measurements performed on MWNT-COOH show increased oxygen content after oxidation of the unpurified MWNTs through increased O—C bonds from 0.5 to 8 wt% and O=C bonds from 0.7 to 8.5 wt% (Table I). Therefore, the physicochemical properties of the MWNT sample are distinctly different from the MWNT-COOH sample. In this regard, it is critical to maintain a careful balance between the surface chemical functionalization of CNTs and their structural integrity in order to retain their novel properties.

In order to assess if there is a differential response of human lung macrophages to unpurified MWNTs compared to acid purified MWNT-COOH, changes in cytotoxicity, morphology and reactive oxygen species (ROS) generation were investigated. Changes in viabilities related to cytotoxicity were spectrophotometrically assayed with the MTS assay, which is sensitive to mitochondrial function. The positive control, CdO was used to verify the validity of the MTS assay and shows a strong and significant decrease in cell viability at the low dose of 5 $\mu\text{g/ml}$ (Fig. 2). By comparison, cells exposed to MWNTs or MWNT-COOH at concentrations ranging from 5–50 $\mu\text{g/ml}$ for 24 h only displayed a significant decrease in viability at the highest concentration of 50 $\mu\text{g/ml}$ for MWNT-COOH compared to no significant decreases in viability at the other concentrations (5–25 $\mu\text{g/ml}$) or at any of the concentrations (5–50 $\mu\text{g/ml}$) for the unpurified MWNTs (Fig. 2).

One explanation for the decreased viability of the cells after exposure to the 50 $\mu\text{g/ml}$ concentration of

Table I. XPS results for oxygen (O) and iron (Fe) content (wt%) of MWNT and MWNT-COOH.

Sample	O				Fe
	H ₂ O	O—C	O=C, —OH	O—Fe	
MWNT	—	0.5	0.7	0.2	0.2
MWNT-COOH	0.8	8.0	8.5	—	<0.1

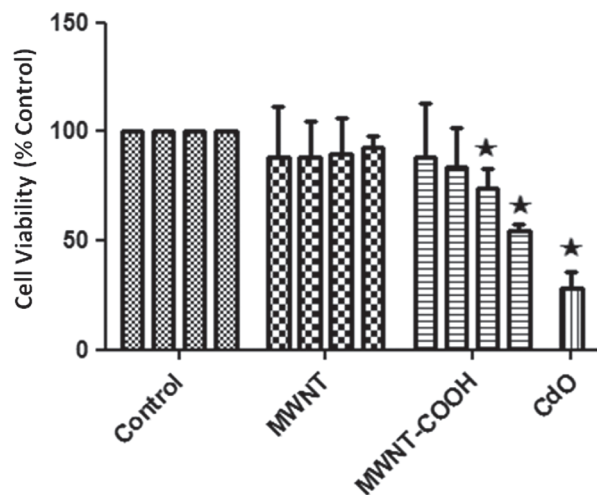


Fig. 2. Cell viability assessment with the MTS assay after 24 h of exposure to MWNTs or MWNT-COOH. There was significant toxicity for the positive control CdO at 5 µg/ml and MWNT-COOH at 50 µg/ml. The data are represented as the average of three independent experiments \pm the standard deviation. Stars indicate significant difference from control $p < 0.05$. Doses are 0 for the Control and 5, 10, 25 and 50 µg/ml for treatment groups.

MWNT-COOH is the difference in surface chemistry between the two samples. In support of this notion, our previous research found that functionalization of MWNTs with sodium sulfuric acid salt ($-\text{SO}_3\text{Na}$ or $-\text{phenyl-SO}_3\text{Na}$) increased CNT biocompatibility in neuroblastoma cells compared to unfunctionalized MWNTs or carboxylic acid ($-\text{COOH}$) functionalized MWNTs.¹³ Further, several other groups have shown similar trends for acid-purified MWNTs where MWNT toxicity was highly dependent upon surface chemistry ($-\text{COOH}$, $-\text{OH}$).^{17–19} Magrez et al., (2006) found that the cell viabilities of human lung tumor cells (H596) were decreased after incubation with MWNTs containing acidic surface functionalities ($-\text{COOH}$, $-\text{OH}$) compared to MWNTs.¹⁸ Bottini et al. (2006) examined MWNTs (diameter = 20–40 nm, length = 1–5 µm, purity >95%) and acid-purified MWNT-COOH at concentrations of 40 µg/ml and 400 µg/ml from 24–120 h in T-cells, then assessed cell viability and proliferation with Trypan Blue dye staining. There were no significant decreases in viability for either material after 24 h. However, after 48 h, viability was significantly decreased for cells incubated with 400 µg/ml concentration of MWNT-COOH compared to the later time point of 96 h for the MWNTs suggesting that MWNT-COOH were more toxic than the MWNTs. At the concentration of 40 µg/ml of MWNT-COOH, western blotting with an antibody against phosphotyrosine demonstrated no detrimental effects on receptor-induced T-cell activation. Thus, 40 µg/ml of MWNT-COOH did not seem to have toxic effects on the function of T-cells, supporting the notion that this amount does not measurably harm the cells.¹⁷ Furthermore, there is a strong relationship between surface chemistry and nanomaterial

dispersion in solution. For example, acid treatment leads to the oxidation of the CNT surface, and the generation of carboxyl ($-\text{COOH}$) and hydroxyl ($-\text{OH}$) groups, which increases their hydrophilicity and dispersion.¹⁸ To eliminate the potential that the CNTs were interfering with the MTS assay, an acellular MTS assay was performed. The results indicated that both MWNTs and MWNT-COOH do not interfere with the chemicals (data not shown).

We previously demonstrated that CNTs have the ability to generate reactive oxygen species (ROS) before and after introduction into certain mammalian cells.²⁸ For this reason, we investigated the ROS response of U937 cells in addition to the acellular response to MWNTs and MWNT-COOH with the fluorescent DCFH-DA assay. The validity of the assay was assessed with the positive control hydrogen peroxide (H_2O_2), which showed a significant increase in ROS at a 2 mM concentration after 24 h (Fig. 3). By comparison, cells exposed to MWNTs at concentrations >25 µg/ml produced a significantly greater ROS response than the controls or cells exposed to MWNT-COOH, which had uniform and low ROS values at all of the concentrations tested (5–50 µg/ml) (Fig. 3). To determine whether the ROS was directly produced by the cells, we performed an acellular ROS assay to assess the ability of the MWNTs or MWNT-COOH to generate ROS in the absence of cells. Neither MWNTs nor MWNT-COOH produced inherent ROS after 24 h at the high 50 µg/ml concentration (data not shown). Therefore, in the absence of cells, there was no significant production of ROS and the results demonstrate a purely cellular response to the MWNTs and MWNT-COOH as we have previously demonstrated in other cells lines.²⁸ These results also

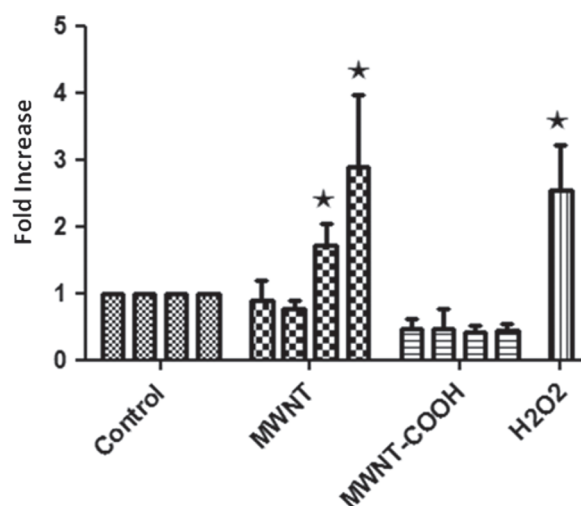


Fig. 3. Assessment of reactive oxygen species (ROS) generation after 24 h showing significant increase after dosing with positive control hydrogen peroxide at 2 mM and MWNTs at 25 and 50 µg/ml. The data are represented as the average of triplicate experiments \pm the standard deviation. Stars indicate significant difference from control $p < 0.05$. Doses are 0 for the Control and 5, 10, 25 and 50 µg/ml for treatment groups.

suggest that the metal catalyst content is directly proportional to the cellular ROS generation by the cells with MWNTs (8–11 wt% Fe) displaying significant ROS levels at 25–50 $\mu\text{g/ml}$ compared to a low ROS response by the cells exposed to the acid-purified MWNT-COOH (0.2–0.49 wt% Fe).²⁸

Indeed, elemental impurities such as residual metal catalysts (Fe, Co, Ni, etc.) from the CNT synthesis process have been implicated in the ROS and toxic response of CNTs.^{16, 20–23} Impure SWNTs (amorphous carbon <5 wt%, Co 2.8 wt%, Cu 0.03 wt%, Fe 0.009 wt%, Mo 4.2 wt%) induced ROS after interaction with A549 cells thereby inhibiting cell growth.^{20, 24} This ROS induction was absent in metal-reduced CNT samples (HNO_3 acid treatment, reduced metal content from 8 wt% to 2.5 wt%) at any time (10 min, 24 h) or concentration (5, 10, 50, 100 $\mu\text{g/ml}$).²⁰ However, the actual amount of residual catalyst may be more important. For example, the present of metal catalyst impurities in MWNTs (impurities 4.24 wt% of Fe, purified 0.08 wt% of Fe) did not influence cytotoxicity (MTT assay, LDH assay, XTT assay) on human lung alveolar epithelial cells (A549).⁷ In this case, the CNTs demonstrate good biocompatibility in macrophages when highly purified.⁸

In many of our previous studies, the production of ROS has been directly linked to nanomaterial toxicity.^{25, 26} However, in this study, U937 cells maintained high viability (MTS assay) even after exposure to high concentrations (50 $\mu\text{g/ml}$) of unpurified MWNTs, while concurrently producing significant levels of ROS. The presence of the Fe catalyst particles likely plays a role in the MWNT-induced ROS response, as mentioned above. Another possible explanation for the reduced viability in the cells incubated with the high dose (50 $\mu\text{g/ml}$) of MWNT-COOH is that the macrophages are able to directly encounter and engulf a greater quantity of the acid-purified and hydrophilic MWNTs-COOH compared to the unpurified and hydrophobic MWNTs due to better dispersion of MWNT-COOH in the cell culture media compared to MWNTs. Support for this mechanism was readily apparent

even at low doses (5 $\mu\text{g/ml}$) from light microscope images of the cells and CNTs (Fig. 4). After 24 h, evidence for uptake of both MWNTs (Fig. 4(B)) and MWNT-COOH (Fig. 4(C)) and subsequent morphological alterations were apparent. However, the cells dosed with MWNT-COOH displayed greater uptake and more irregular borders and enlarged morphologies than the cells dosed with MWNTs. This suggests that, indeed, better dispersion can lead to cellular alterations such as increased uptake and toxicity, which may or may not be detectable by biochemical assays (i.e., MTS, ROS), which rely on large cell numbers and averages. In support of our results, a recent study by Magrez et al. (2006) demonstrated with light microscopy and cytopathological analysis that human lung tumor cells (H596) after 24 h of incubation with very low doses (0.02 $\mu\text{g/ml}$) of MWNTs lose their mutual attachments, retract their cytoplasm and have smaller and more condensed nuclei. However, these distinct morphological changes would not have been noticed from the very slight differences at this same time point and concentration when measured with the MTT cell viability assay. A different study demonstrated that grinding MWNTs can lead to increased dispersion and cell availability leading to a higher cytotoxic and pro-inflammatory response in rat peritoneal macrophages, suggesting that dispersion can have a direct impact on cytotoxicity.⁴ Therefore, thorough physicochemical characterization (size, morphology, surface chemistry, etc.) of the materials prior to cell culture studies⁸ in conjunction with examination of the macroscopic properties of nanomaterials under cell culture conditions and at the level of individual cells provides a critical dimension for understanding the results.²⁷

3. SUMMARY AND CONCLUSIONS

Although MWNTs and MWNT-COOH can accumulate in human lung macrophage cells to different degrees, they do not produce overt cell toxicity under these conditions (5–50 $\mu\text{g/ml}$ MWNT or MWNT-COOH, 2–24 h). However, there are morphological alterations at

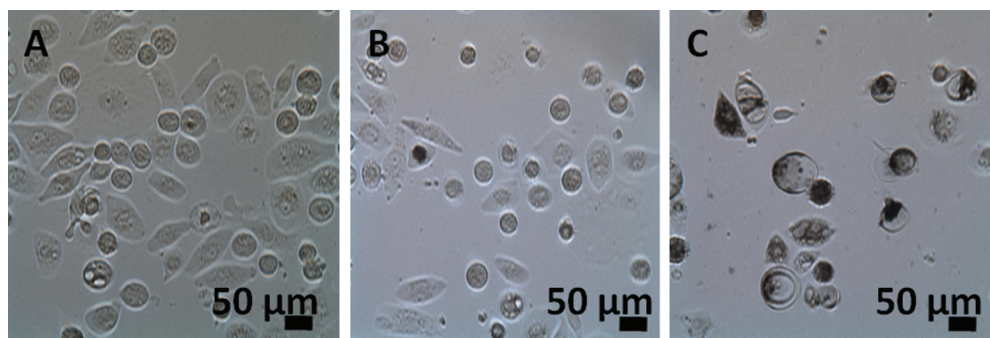


Fig. 4. Light microscope morphological and uptake assessment of cells exposed to 5 $\mu\text{g/ml}$ MWNTs or MWNT-COOH for 24 h. (A) Control (B) 5 $\mu\text{g/ml}$ MWNTs or (C) 5 $\mu\text{g/ml}$ MWNT-COOH for 24 h. Notice that the cells dosed with MWNT-COOH show greater uptake and more irregular morphologies than the cells dosed with MWNTs. Original images taken at 20 \times magnification. Scale bars are 50 μm .

Table II. Summary of results.

Observation/ Assay	Description	MWNTs	MWNT-COOH
Extent of agglomeration	Light microscopy	High	Low
Residual metal catalyst (Fe) Content	Materials characterization	High (11 wt%)	Low (1.9 wt%)
Surface chemistry	Degree of oxidation	Low (0.7 wt% O=C and -OH)	High (8.5 wt% O=C and -OH)
Macrophage uptake	Light microscopy	Yes	Yes (greater than MWNTs)
MTS	Cell viability, 24 h	No significance	Significant decrease at 25, 50 $\mu\text{g/ml}$
ROS	Reactive oxygen species generation, 24 h	Concentration-dependent increase from 25–50 $\mu\text{g/ml}$	Low at all concentrations from 5–50 $\mu\text{g/ml}$

low doses of MWNT-COOH and significant ROS production for MWNTs at high doses indicating a potential, although different, cellular stress response for both materials (Table II). Therefore, before nanomaterials are fully accepted and integrated into biological systems, they will continue to undergo further scrutiny at various stages of their processing (i.e., before and after purification) and with models ranging from simple to complex (i.e., cells vs. whole animals) to gain a better understanding between their physicochemical properties and bio-effects.

4. MATERIAL AND METHODS

4.1. Carbon Nanotube Characterization

The commercially-available multi-walled carbon nanotubes (MWNTs) were synthesized by pyrolysis of propylene using iron-based catalyst were purchased from Tsinghua and Nanfeng Chemical Group Cooperation, China and were previously used for DNA damage and nanotoxicity studies.^{16, 28, 29} In order to remove the catalyst residues and generate carboxylic acid surface functional groups, the as-received multiwall carbon nanotube (MWNT) was refluxed with vigorous stirring in a sulfuric acid (H_2SO_4)/nitric acid (HNO_3) mixture (3:1, V:V) for 3 hrs at 100 °C. After cooling to room temperature, the acidic solution was poured into ice water. The aqueous black suspension was filtered through a 0.45 μm nylon membrane and washed repeatedly with excess water. Finally, the purified MWNTs were dried under vacuum overnight. Transmission electron microscopy was performed on a Hitachi H-7600 TEM at 100 kV after spotting the MWNTs onto formvar/carbon coated grids. The air thermogravimetric analysis was performed on a TGA TA500. X-ray photoelectron spectroscopic (XPS) measurements were performed on a VG

Microtech ESCA 2000 using monochromatic Mg K radiation at a power of 300 W). Raman spectra were obtained on an inVia micro-Raman spectrometer (Renishaw), recorded with a 514 nm laser for nanotubes. MWNT stock solutions were prepared at concentrations of 1 mg/ml in water, then briefly sonicated for 2–3 minutes with intermittent rest for ~3 minutes and repeated 3–4 times.

4.2. Cell Culture

Human alveolar macrophage cells (U937, ATCC) were culture in T-75 flasks and incubated with 5% CO_2 at 37 °C. The U937 cells are a monocyte cell line that can be stimulated to mature into macrophages, which play the major role in both non-specific and specific defense mechanisms in the body. The U937 cells were maintained in RPMI media supplemented with 10% Heat Inactivated FBS (Invitrogen) and 1% penicillin and streptomycin (Invitrogen). The cells were stimulated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA) for 48 hours to allow differentiation into human alveolar macrophage cells before dosing.

4.3. MTS Assay

The MTS assay (Promega) was used to investigate mitochondrial function. Yellow MTT (3-(4,5-dimethyliazol-2-yl)-(2,5-diphenyltetrazolium bromide) is reduced to aqueous insoluble purple formazan by a mitochondrial enzyme showing intact functional mitochondria. Mitochondrial function of the cells was spectrophotometrically investigated after 24-h exposure to MWNTs and MWNTs-COOH. Briefly, the culture media was removed, and fresh media without FBS containing a 1:10 dilution (20 μl MTS solution: 200 μl exposure medium) was added and incubated for 2 hours. A spectrophotometer (BioTek Synergy HT, USA) was used to determine the absorbance at 490 nm, according to the manufacturer's procedure. The data are represented as the average of triplicate \pm the standard deviation.

4.4. Light Microscopy

Cells were observed with light microscopy (Olympus phase contrast microscope) to assess overall changes in morphology and MWNT uptake. U937 cells were cultured in six well dishes at a density of 4.2×10^4 cell/ml, then stimulated with PMA (100 ng/ml) for 48 hours. After washing with 1xPBS (Invitrogen), the solution was changed to RPMI medium, dosed with MWNT and MWNT-COOH and MWNTs at final concentrations of 5 $\mu\text{g/ml}$.

4.5. Reactive Oxygen Species (ROS) Generation

For the ROS assay-DCFH-DA, U937 cells were seeded at density 150×10^3 /ml in black 96 well plates. U937 cells

were stimulated with PMA at 100 ng/ml for 48 hours, then the media was removed the cells were washed with 1xPBS (Invitrogen) twice. 200 μ l of 100 μ M DCFH-DA (Invitrogen) (in culture medium) was added to each well and incubated at 37 °C and 5% CO₂ culture conditions for 30 minutes. After the aspiration of the DCFH-DA, 200 μ l of dosing solution (5, 10, 25, 50 μ g/ml) was added to each well. The positive control hydrogen peroxide (Fisher Scientific) was applied at a concentration of 2 nM. The plate was covered with aluminum foil to block light and placed in an incubator for 24 hours, then the intensity of the ROS probe was measured on a spectrophotometer (BioTek, Synergy HT) with excitation at 485/20 nm and absorbance at 528/20 nm, according to the manufacturer's procedure. The data are represented as the average of triplicate \pm the standard deviation.

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